PRODUCTION OF BIOSURFACTANT FROM LOCALLY ISOLATED BACTERIA

RIHAB HUSSEIN JAWAD

Thesis submitted in fulfilment of the requirements
for the award of the degree of
Master of Engineering in (Bio-process)

Faculty of Chemical & Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

AUGUST 2010

ABSTRACT

In this study, a total of 176 isolates was obtained from two coastal sampling locations. Out of this total, 124 (70.4%) isolates were obtained from the seawaters of the coast of Kertih, Terengganu while the remaining 52 isolates (29.5 %) were from Kuantan, Pahang. Five bacterial strains previously isolated were selected for the screening of biosurfactant producer(s) via three different characterization tests for biosurfactant; (i) surface tension measurements, (ii) emulsification activity, and (iii) cetyltrimethylammonium bromide assay (CTAB) test. One isolate coded KRT-142 identified as *Pseudomonas aeruginosa* was chosen to be the best candidate for biosurfactant production. Biosurfactant productions by isolated bacteria were found to be growth-associated in all the conditions tested. Microbiological properties of strain KRT-142 were investigated. It was found that strain KRT-142 produces water soluble, greenish yellow fluorescent pigments on a nutrient agar plate. It is an aerobic, gram negative, straight rods, motile bacteria, and not surrounded by sheaths. Ethanol as a carbon source was found to support the highest growth (as measured by whole cell protein) followed by glycerol and glucose. Slight growth was also observed with crude oil. Decreasing growth was observed with tetradecane, 1-propanol, 1-butanol, sucrose and maltose. Ethanol yielded maximum biosurfactant production, reducing the surface tension to 43.3 mN/m. It was followed by glycerol, hexadecane and crude oil with surface tension reduction to 44.5, 49 and 53.5 mN/m, respectively. The highest emulsifying activity was 56% at 7h and 52.7% at 14h for ethanol. In the study of organic nitrogen sources, soytone supported the highest growth followed by peptone, meat extract, yeast extract, tryptone and casamino acid, Soytone yielded the highest biosurfactant production, followed by meat extract and tryptone. At the optimum conditions (35^{o}C, 4% inoculum size, 100 rpm and pH 7.2), the surface tension reached a minimum of 30.76 mN/m, after 6h in the stationary growth phase. Stable and compact emulsification index (E24) was observed after 2h of cultivation, reaching a maximal value of 86% at 6h of incubation.

ABSTRAK

Dalam kajian ini sebanyak 176 isolat telah diperolehi dari 2 lokasi pesisir pantai. Daripada jumlah tersebut, 124 (70,4%) isolat diperolehi dari pesisir Kertih, Terengganu manakala 52 isolat (29,5%) lagi di ambil dari pantai berdekatan Kuantan, Pahang. Lima jenis bakteria yang telah dipencilkan dipilih untuk proses saringan bagi penghasilan biosurfaktan melalui tiga kaedah pencirian biosurfaktan iaitu (i) pengukuran ketegangan permukaan (ii) aktiviti pengemulsian, dan (iii) ujian assay cetiltrimetilammonium bromida (CTAB). Isolat kod KRT-142 yang dikenalpasti sebagai *Pseudomonas aeruginosa* dipilih sebagai calon terbaik bagi penghasilan biosurfaktan. Penghasilan biosurfaktan oleh isolat bakteria didapati berkait rapat dengan pertumbuhannya pada semua keadaan yang diuji. Kajian terhadap sifat mikrobiologi strain-142 KRT juga telah dijalankan. Strain KRT-142 didapati menghasilkan pigmen fluorescent kuning-hijau yang larut air di atas nutrien agar. Ia merupakan bakteria aerobik, gram negatif, berbatang lurus dan motil. Ia juga tidak dikelilingi oleh selubung. Etanol, sebagai sumber karbon, didapati menyokong pertumbuhan paling tinggi apabila protein sel keseluruhan diukur. Ini diikuti oleh gliserol dan glukosa. Sedikit pertumbuhan juga didapati apabila menggunakan minyak mentah. Penurunan pertumbuhan didapati dengan penggunaan tetradekana, 1-propanol, 1-butanol, sukrosa dan maltosa. Etanol menghasilkan pengeluaran biosurfaktan tertinggi, mengurangkan tegangan permukaan kepada 43.3 mn/m. Ini diikuti oleh gliserol, minyak mentah dan heksadekana, dengan pengurangan ketegangan permukaan 44.5, 49 dan 53.5 mn/m masing-masing. Untuk etanol, Aktiviti pengemulsian paling tinggi, iaitu sebanyak 56% didapati pada 7 jam, dan 52.7% pada 14 jam. Untuk kajian sumber nitrogen organik, soyton didapati menyokong pertumbuhan paling tinggi, diikuti oleh pepton, ekstrak daging, ekstrak ragi, trypton dan asid kasamino. Soyton menghasilkan pengeluaran biosurfaktan paling tinggi, diikuti oleh ekstrak daging dan trypton. Pada keadaan optima (35^{o}C, 4% saiz inokulasi, 100 rpm dan pH 7.2), ketegangan permukaan mencecah ke tahap minima, iaitu 30.76 mN/m, selepas 6 jam berada dalam fasa pertumbuhan malar. Indeks pengemulsian (E24) stabil dan padat dicapai setelah kultivasi selama 2 jam, dan mencapai nilai maksimum 86% bagi tempoh pengeraman 6 jam.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

$CaCI_2$	Calcium chloride
cm	Centimetre
CMC	Critical micelle concentration
d	Day
dH_2O	Distilled water
g	Gram
g/L	Gram per liter
HCI	Hydrochloric acid
h	Hour
L	Liter
M	Molar
mg	Milligram
mL	Millilitre
mM	Millimolar
mm	Millimeter
NaC1	Sodium chloride
Na_2HPO_4	Disodium hydrogen orthophosphate
NaOH	Sodium hydroxide
SDS	Sodium dodecyl sulphate
PAHs	Polycyclic-Aromatic-Hydrocarbons
R^2	Coefficient of determination
Rpm	Round per minute

TLC	Thin layer chromatography
TSB	Trypticase Soy Broth
μg	Microgram
μL	Microlitre
μm	Micrometer
v/v	Volume per volume
w/v	Weight per volume

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 BACKGROUNDS OF SURFACTANT AND BIOSURFACTANT

Surfactants are amphiphilic compounds that reduce the free energy of the system by replacing the bulk molecules of higher energy at an interface (Mulligan, 2005). It contains a hydrophobic moiety with little affinity for the bulk medium and a hydrophilic portion that is attracted to the bulk medium. Surfactants have been used industrially as flocculating, wetting, foaming agents, adhesives and deemulsifiers, lubricants and penetrants (Mulligan and Gibbs, 1993). The ability to reduce surface tension is a major characteristic of surfactant. Because of their amphiphilic nature, surfactants tend to accumulate at interfaces (air-water and oil-water) and surfaces. As a result, surfactants reduce the forces of repulsion between unlike phases at interfaces or surfaces and allow the two phases to mix more easily (Bodour and Miller-Maier, 2002). Due to the presence of surfactant, less work is required to bring a molecule to the surface, and the surface tension is reduced. It is obvious that their surface and membrane-active properties play an important role in the expression of their activities. Commercially, Surfactants are key ingredients used in detergents, shampoos, toothpaste, oil additives and a number of other consumers and industrial products..

Biosurfactant is a structurally diverse group of a surface-active molecule synthesized by microorganisms. Their capability to reduce surface and interfacial tension with low toxicity and high specificity and biodegradability, lead to an increasing interest on these microbial products as alternatives to chemical surfactants (Banat et al., 2000). Hester (2001) estimated that biosurfactants could capture 10% of the surfactant market by the year 2010 with sales of $US200 million.

However, up to now, biosurfactants is still unable to compete with the chemically synthesized surfactants in the surfactant market. This could be due to their high production costs in relation to inefficient bioprocessing method available, poor strain productivity and the need to use expensive substrates (Cameotra and Makkar, 1998; Deleu and Paquot, 2004).

The interest in biosurfactant has been steadily increasing in recent years due to the possibility of their production through fermentation and their potential applications in such areas as the environmental protection. The uniqueness with unusual structural diversity, the possibility of cost-effective ex-situ production and their biodegrability are some of the properties that make biosurfactant a promising choice for use in environmental application (Hua et al., 2003). Initial focus of industrial interest towards biosurfactants concentrates on the microbial production of surfactants, cosurfactants and so on for the application on microbial-enhanced oil recovery (MEOR)(Thomas, 2008). The applications of biosurfactants however, are still at the developmental stage of industrial level. The development of biosurfactant application in industries is focused mainly on high biosurfactant production yield and the production of highly active biosurfactants with specific properties for specific applications.

Majority of surfactants produced today are of petrochemical origin beside from renewable resources like fats and oils (Deleu and Paquot, 2004). Amongst the renewable raw materials, oleochemical products represent half of the total surfactant production. The petrochemical industry is one of the important sectors in Malaysia, with investments totalling RM34.8 billion as at the end of 2008 (MIDA, 2009). Unfortunately, industrial wastewater from petroleum-related industries has been identified as one of the major sources of pollution in Malaysia. The biodegradation of a petroleum pollutant and its related compound is limited by poor availability to the microorganisms, due to their hydrophobicity and low aqueous solubility. This suggests that by applying biosurfactants to influence the bioavailability of the contaminant can possibly enhance the solubility of these compounds. Due to their biodegradability and low toxicity, they are in demand to be use in remediation technologies (Mulligan, 2004).

Biosurfactants plays an important application in petroleum-related industries which such as enhanced oil recovery, cleaning oil spills, oil-contaminated tanker cleanup, viscosity control, oil emulsification and removal of crude oil from sludges (Daziel et al., 1996, Bertrand et al., 1994). These industries are known to be the potential target for the application of these compounds. This is due to the ability of biosurfactant-producing microorganisms to use petroleum or its' products as substrates as well as the properties of the biosurfactant which require less rigorous testing than chemical surfactant, there are numbers of reports on the synthesis of various types of biosurfactants by microorganisms using water-soluble compounds such as glucose, sucrose, ethanol or glycerol as substrates (Desai and Banat, 1997).

Sea water was found to be a great potential in producing a microorganism that may produce biosurfactants (Maneerat and Phetrong, 2007). Hence, there could probably be a potential chance of producing biosurfactants using locally isolated bacteria originated from sea water available in this country. It has been focused here that improving the method of biosurfactant production and characterizing the major properties of the biosurfactant are highly important in the commercial application of biosurfactant.

1.2 PROBLEM STATEMENT

Many factors affecting on the production of the surface-active molecules of biological origin, such as the type and amount of the microbial surfactants produced, which depend primary on the producer organism, factors like carbon and nitrogen, trace elements, temperature, and aeration also affected their production by the organism.

Many of the potential applications that have been considered for biosurfactants depend on whether they can be produced economically; however, much effort in process optimization and at the engineering and biological levels have been carried out. In addition, legal aspects such as stricter regulations concerning environmental pollution by industrial activities and health regulations will also strongly influence the chances of biodegradable biosurfactants replacing their chemical counterparts. Aiming at the final biosurfactant cost reduction, the development of economical alternatives for its production has been investigated. Thus, the use of low-cost raw matter appears as a natural choice to generate an overall economy.

Pollution of the sea, especially by crude oil, which is caused by stranding of tankers, is one of the serious environmental problems over the world. The operations of the ships also produce wastes, this waste must be managed properly to avoid environmental pollution. Biodegradation by marine microorganisms is overburdened due to the additional hydrocarbons, especially large oil spills. Therefore, the use of biosurfactants can be playing an important role by emulsifying the polluted oils prior to biodegradation. Due to the long coasts of South China Sea, Strait of Malacca and Strait of Johor, the importance biodiversity in the sea has been recognized. However, no information regarding the biosurfactant-producing marine bacteria has been reported in Malaysia.

1.3 OBJECTIVES

The objective of this study is to:

1- Screening for biosurfactant producing bacteria
2- Characterizing the selected bacteria producing biosurfactant.
3- Production of biosurfactant by bacteria isolated for a potential biosurfactant production.
4- Optimization of biosurfactant productions where only one parameter is varied at any one time with the others being kept constant and interactions the parameters to set optimal conditions.

1.4 SCOPES

The principal scope of the experimental work was therefore, to develop optimize, and purify the biosurfactant production by local marine bacteria. Such a programme of product and process developments entailed several stages, which are:

1- Screening test and characterization of the potential biosurfactant-producing microbes from sea water samples using various screening methods.
2- Examining the effect of nutritional and physical parameters on the biosurfactant production by isolated bacteria.
3- Chosing the best substrate for comorcial production.
5- Obtaining a set of optimal conditions for the production.
6- Conducting optimized production.
7- Recovery of biosurfactant production.
8- Analysis of biosurfactant production by thin layer chromatography (TLC).

1.5 RESEARCH CONTRIBUTIONS

This study investigated the potential production of biosurfactants using locally isolated bacteria originating from sea water. It has been focused here that improving the optimizatin of biosurfactant production and the major factor's effect on production, which were highly important in the commercial production of biosurfactant.

1.6 THESIS ORGANIZATIONS

This thesis consists of five main chapters, including an introduction in Chapter 1. The literature related to classification, chemical nature of biosurfactant, factors affecting biosurfactant production, recovery and applications of biosurfactants are discussed in Chapter 2 while, the methodology, apparatus and equipment for experimental work are discussed in Chapter 3. In addition, the experimental results are discussed in Chapter 4, and the conclusion and recommendations are summarized in last chapter, which is Chapter 5. This thesis is completed with references and appendices.

CHAPTER 2

LITERATURE REVIEW

A review of previous studies relevant to biosurfactants production was conducted. The classifaction and chemical nuture of surfactant are decumenated with different type of microorganisms related to production of biosurfactants. The biosurfactant production study was an introduction of this chapter followed by classification and chemical nature of biosurfactants and the factors such as nutritional and physical affecting on production. Finally, the potential applications of microbial surfactant discussed in detail in this chapter.

2.1 INTRODUCTION

All living cells produce amphipathic molecules. These molecules which consist of both hydrophilic and hydrophobic moieties are called surface-active compounds or surfactants. In many cases, they exhibit surface-active characteristics such as dramatic lowering of surface tension at the air/water interface, lowering interfacial tension at the oil/water interface, and micelle or pseudomicelle formation (Haddad et al., 2008). Such characteristics confer excellent detergency, emulsifying, foaming, and dispersing traits, which make surface-active compound, some of the most versatile process chemicals (Greek, 1991).

Microorganisms utilize a variety of organic compounds as the source of carbon and energy for their growth. When the carbon source is an insoluble substrate like a hydrocarbon (CxHy) microorganism facilitate their diffusion into the cell by producing a variety of substances, the biosurfactants. Some bacteria excrete ionic surfactant, which emulsify hydrocarbon substrates in the growth medium. The exact reason why some microorganisms produce surfactant is unclear (Deziel et al., 1996). Biosurfactants produced by various microorganisms together with their properties are listed in Table 2.1.

Table 2.1: Structural Types of Microbial Surfactants

Biosurfactant	Source
Glycolipids	
Trehalolipids	*Rhodococcus erythropolis,* *Nocardia erythropolis*
Trehalose Dimycolates	*Mycobacterium sp., Nocardia sp.*
Trehalose dicorynemycoaltes	*Arthrobacter sp., Corynebacterium sp.*
Rhamnolipids	*Pseudomonas aeruginosa* *Pseudomonas sp.*
Sophorolipids	*Torulopsis bombicola, Torulopsis Apicola,* *Torulopsis petrophilum Torulopsis sp.*
Cellobiolipids	*Ustilago zeae, Ustilago maydis*
Aminoacid-lipids	*Bacillus sp.*
Lipopeptides and lipoprotein	*Streptomyces sp., Corynebacterium sp.,* *Mycobacterium sp.*
Peptide-lipid	*Bacillus licheniformis*
Serrawettin	*Serratia marcescens*
Viscosin	*Pseudomonas fluorescens*
Surfactin	*Bacillus subtilis*
Subtilisin	*Bacillus subtilis*
Gramicidins	*Bacillus brevis*
Polymyxins	*Bacillus polymyxa*
Ornithine-lipid	*Pseudomonas sp., Thiobacillus sp.* *Agrobacterium sp., Gluconobacter sp.*
Phospholipids	*Candida sp., Corynebacterium sp.* *Micrococcus sp., Thiobacillus sp.*
Fatty acids /Natural lipids	*Acinetobacter sp.,Pseudomonas sp.,* *Micrococcus sp., Mycococcus sp.,* *Candida sp., Penicillium sp.,* *Aspergillus sp.*
Polymeric surfactants	
Emulsan	*Arethrobacter calcoaceticus*
Biodispersan	*Arethrobacter calcoaceticus*
Mannan-lipid-protein	*Candida tropicalis*
Liposan	*Candida lipolytica*
Carbohydrate-protein-lipid	*Pseudomonas fluorescens* *Debaryomyces polymorphis*
Protein PA	*Pseudomonas aeruginosa*
Particulate biosurfactants	
Vesicles and fimbriae Whole cells	*Arthrobacter calcoaceticus*

(Muthusamy et al., 2008)

2.2 CLASSIFICATION AND CHEMICAL NATURE OF BIOSURFACTANTS

Biosurfactants are categorised mainly by their chemical composition and their microbial origin. The microbial surfactants are complex molecules covering a wide range of chemical types, including glycolipids, peptides, fatty acid, phospholipids, antibiotics and lipopiptides. Microorganisms also produce surfactants that are in some cases' combination of many chemical types referred to as the polymeric microbial surfactants. . A broad classification of biosurfactants is given in Table 2.2.

2.2.1 Glycolipids

The low molecular weight biosurfactants are generally glycolipids or lipopeptides (Table 2.1). The best studied glycolipid bioemulsifiers, rhamnolipids, trehalolipids and sophorolipids, are disaccharides that are acylated with longchain fatty acids or hydroxy fatty acids (Rosenberg, 2006). The constituent monosaccharides, disaccharides, trisaccharides and tetrasaccharides include glucose, mannose, galactose, glucuronic acid, rhamnose, and galactose sulphate. The fatty acid component usually has a composition similar to that of the phospholipids of the same microorganism. The glycolipids can be categorized as:

i. Trehalose lipids

The serpentine growth seen in many members of the genus *Mycobacterium* is due to the presence of trehalose esters on the cell surface. A succinoyl trehalose lipid produced by *Rhodococcus* sp. behaves as a biological surfactant and also displays various interesting biological activities (Zaragoza et al., 2010**).** Yields of trehalose lipids were increased to 4 g/liter when the bacteria were grown on 10% (w/v) n-alkanes and the trehalose lipids were continuously extracted. The yield of rhamnolipids was increased to 24.3 g/liter in media containing 6% canola oil (Sim et al., 1997). Trehalose mycolates reduced the surface tension of water from 72 to 26 mN/m (Lang and Philip, 1998).

Table 2.2: Classification of Biosurfactant

Biosurfactant	Type
1-Glycolipids	Trehalose lipids Sophorolipids Rhamnolipids
2-Fatty acids	
3-Phospholipids	
4- Lipopeptides antibiotics	Gramicidin Polymixins Surfactine
5-Polymeric microbial surfactants	Emulsan from *Acinebacter calcoacceticus* RAG-1 (ATCC 31012). The polysaccharide protein complex of *Acinebacter Calcoaceticus* BD4. Other *Acinetobacter* sp. Emulsifiers Emulsifing protein from *Pseudomonas aeruginosa.* Emulsifying and solubilizing factors from *Pseudomonas* sp. PG-1. Bioflocculant and emulcyan from the filamentous *Cyanobacterium phormidium* J-1.
6-Particulate surfactant	Extracellular vesicles from *Acinetobacter* sp. HO1-N. Microbial cell with high cell surface hydrophobicities.

(Muthusamy et al., 2008)

ii. Sophorolipids

These are produced by different strains of the yeast, the sugar unit is the disaccharide sophorose which consists of two β-1, 2-linked glucose units, the 6 and 6' hydroxy groups are generally acetylated. *Candida apicola* and *Candida bombicola* produced extracellular sophorolipids biosurfactant which was a mixture of acidic and lactonic forms (Thaniyavarn et al., 2008). The sophorolipids reduce surface tensions between individual molecules at the surface, although they are effective emulsifying agents (Hirata et al., 2009). The sophorolipids of *Torulopsis* have been reported to stimulate, inhibit and have no effect on growth of yeast on water-insoluble substrates. The yields have improved to over 150 g/liter (Davila et al., 1997).